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TOXINS PRODUCED BY BENTHIC DINOFLAGELLATES

TAKESHI YASUMOTO*, NAOKO SEINO**, YASUTAKA MURAKAMI***, AND MICHIO MURATA

Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Sendai 980, Japan

Abstract

Nine species of benthic dinoflagellates collected in subtropical waters were cultured, extracted, and tested for mouse lethality, ichthyotoxicity, and hemolytic activity. Hemolytic activity was detectable in all species, but the activities of *Amphidinium carteri, A. klebsi,* and *Gambierdiscus toxicus* were outstanding. *G. toxicus* showed the most potent mouse lethality. Two hemolytic constituents of *A. carteri* were determined to be mono- and di-galactoglycerolipids. Maitotoxin, produced by *G. toxicus,* was suggested to have a molecular weight of 3402 ± 2 (*m/z*). Two potent toxins against mice were isolated from *Prorocentrum lima* and identified as okadaic acid and 5-methylene-6-hydroxy-2-hexen-1-okadaate.

Introduction

Scientists first noticed the toxigenicity of benthic dinoflagellates when *Gambierdiscus toxicus* was found to produce and to transmit ciguatoxin and maitotoxin to herbivorous fish (Yasumoto *et al.,* 1977). Subsequent *G. toxicus* distribution surveys revealed an abundance, in terms of both species and population, of benthic dinoflagellates in coral reef communities. This observation suggests that toxic metabolites, if any, of these benthic species are taken up by herbivorous fish, as ciguatoxin is, and contribute to the manifestation of the complex symptoms of ciguatera. The actual occurrence of minor toxins in the viscera of herbivorous fish has been confirmed, and the toxigenicity of several benthic species has also been demonstrated previously (Yasumoto *et al.,* 1976; Nakajima *et al.,* 1981). The present paper briefly summarizes our knowledge of the toxic benthic species and the chemical natures of their toxins; ciguatoxin, however, has been described separately by Tachibana *et al.* (1986).

Materials and Methods

Benthic dinoflagellates collected at Okinawa, Japan, were cultured in a nutrient-enriched seawater medium described by Provasoli (1968). The following nine species were tested for the toxin production: *Amphidinium carteri, A. klebsi, Coolia monotis, Gambierdiscus toxicus, Ostreopsis ovata, O. siamensis, Prorocentrum concavum, P. lima,* and *P. rhathymum.*

The harvested cells were extracted with boiling methanol. The methanol was removed by evaporation, and the residue was then suspended in water and extracted, first with diethyl ether and then with 1-butanol. The residues obtained after evapora-

* To whom correspondence should be addressed.

,* Present addresses: N. Seino, Ohmori Research Institute of Tobishi Pharm. Co. Ltd., 1-16-18 Ohmorinishi, Ohtaku, Tokyo 146, Japan; Y. Murakami, MITSUKAN Co. Ltd. 2-6 Nakamuracho, Handa, Aichi Pref. 475, Japan.

TABLE I

Toxicigenicity of benthic dinoflagellates

Species	Mouse lethality	Ichthyotoxicity	Hemolytic activity
Amphidinium carteri	+	++	++
Amphidinium klebsi	++	++	++++
Coolia monotis	−	−	+
Gambierdiscus toxicus	+++++	−	+++++
Ostreopsis ovata	+	−	+
Ostreopsis siamensis	+++	−	+
Prorocentrum concavum	++	+++	+
Prorocentrum lima	+++	−	+
Prorocentrum rhathymum	−	−	+

The relative potency is expressed by increasing the number of +, the potency of undetectable level is expressed by −.

tion of the solvents were tested, respectively, for mouse lethality, ichthyotoxicity, and hemolytic activity, as described previously (Nakajima *et al.*, 1981).

Three toxins, tentatively named PL toxin-1, -2, and -3, were isolated from *P. lima* by successive treatments on columns of silicic acid ($CHCl_3$-MeOH, stepwise), Sephadex LH-20 ($CHCl_3$-MeOH 2:1), LiChroprep RP-2 (Merck, MeOH-H_2O 2:1), and ODS (Kyowaseimitsu, MeOH-H_2O 4:1).

Five hemolytic compounds (hemolysin-1 to -5) were present in *A. carteri*. Hemolysin-1 and -2 were purified on columns of silicic acid ($CHCl_3$-MeOH, stepwise) and ODS Q-3 (Fujigel, MeOH-H_2O 9:1). Hemolysin-3 to -5 were also purified in a similar manner but further purification on a Toyopearl 40 column was necessary (MeOH-H_2O, stepwise).

Purification of maitotoxin was carried out on columns of silicic acid ($CHCl_3$-MeOH, stepwise), Develosil ODS (Nomurakagaku, MeOH-H_2O, stepwise), and Develosil TMS (MeCN-H_2O 35:65).

^1H NMR and ^{13}C NMR spectra were taken on either a Nicolet NT 360 spectrometer or a JEOL FX-100 spectrometer, and mass spectra were taken on a Hitachi M-80 mass spectrometer.

RESULTS

Bioassays indicate that all the dinoflagellates are toxic by at least one of the assay methods, as shown in Table I. Mouse lethality was observed in *A. carteri, A. klebsi, G. toxicus, O. siamensis, P. concavum,* and *P. lima.* The toxicity of *G. toxicus* to the mouse was outstanding. Hemolytic activity was most prominent in *A. carteri* and *A. klebsi,* although observed in all species tested. Potent ichthyotoxicity was observed in *A. carteri, A. klebsi,* and *P. concavum.*

The chromatographic and spectral analyses of PL toxin-2, the major toxin produced by *P. lima,* proved it to be okadaic acid (Murakami *et al.*, 1982), a cytotoxic polyether fatty acid derivative previously isolated from sponges (Tachibana *et al.*, 1981). PL toxin-1 was found to be a mixture of diol esters of okadaic acid. The structures of okadaic acid and the major ester in the PL toxin-1 fraction are shown in Figure 1. PL toxin-3 was shown to be a tertiary amine having a molecular weight of 981 (*m/z*). Elucidation of its chemical structure is under way.

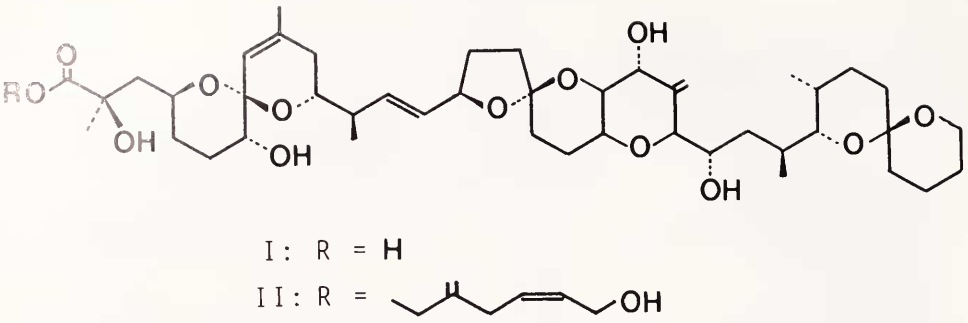

FIGURE 1. Okadaic acid (I) and one if its diolesters (II) from *Prorocentrum lima.*

Among the five hemolysins of *A. carteri,* hemolysin-1 and -2 were more abundant than the other three. The structures of hemolysin-1 and -2 were determined to be O-β-D-galactopyranosyl-(1-1)-3-O-octadecatetraenoyl-D-glycerol and O-α-D-galactosyl-(1-6)-O-β-D-galactopyranosyl-(1-1)-3-O-octadecatetraenoyl-D-glycerol, respectively (Fig. 2). The hemolytic activities of hemolysin-1 and -2 were 80% and 25% of that of the commercial saponin (Merck), respectively. The hemolytic activities of hemolysin-3, -4, and -5 were 100, 9, and 2 times more potent than the commercial saponin, respectively. Their chemical structures were indicated to be entirely different from those of hemolysin-1 and -2. Further structural work is under way.

Maitotoxin judged to be homogeneous by HPLC and TLC was obtained as an amorphous solid. It was extremely lethal to mice (0.13 μg/kg, ip). Mass spectra suggested the molecular weight of 3402 ± 2 (*m/z*). Chemical and spectral analyses indicated the absence of any amino acid or fatty acid moieties in the molecule. Further analyses of the structure are underway.

DISCUSSION

The occurrence of diverse toxins in benthic dinoflagellates was confirmed. Out of the nine species tested, six produced mouse-lethal toxins, three ichthyotoxins, and all species produced hemolytic substances. Such a high occurrence of toxins is a charac-

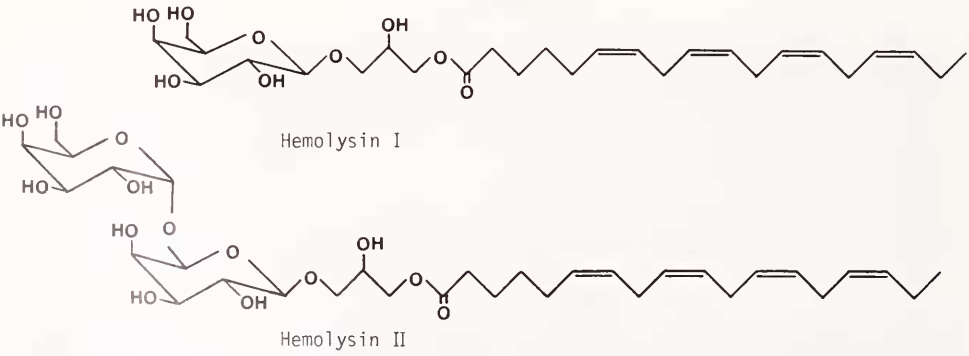

FIGURE 2. Hemolysin I and II from *Amphidinium carteri.*

teristic feature of benthic dinoflagellates. The biological and ecological significance of the toxins are not clear at present. Whether the toxins deter the growth of other microorganisms and thus benefit the elaborators remains to be tested.

The monoacylgalactolipids (hemolysin-1 and -2) are closely related to the known intermediate metabolites of photosynthesis, and therefore are likely to be widely distributed. The compounds may not be involved in ciguatera, but they could be responsible, in part, for fish kills during blooms of dinoflagellate species with no known ichthyotoxins.

Production of okadaic acid by *P. lima* is interesting because of its chemical resemblance to ciguatoxin and its potent diarrheagenicity. As *P. lima* is widely and densely distributed in coral reefs, there is a possibility that the compound, like ciguatoxin, is taken up by herbivorous fish and thus contributes to the diarrhea which is frequently seen in ciguatera patients.

The presence of maitotoxin in the viscera of surgeonfish has already been confirmed, and the toxin has been suspected of contributing to the diverse ciguatera symptoms seen in patients who have eaten herbivorous fish without first eliminating the viscera. The most characteristic feature of maitotoxin is its high lethality to mice (0.13 μg/kg, ip), which is 70 times that of saxitoxin or tetrodotoxin. The specific action of maitotoxin, to enhance the calcium ion influx through excitable membranes, was first discovered by Ohizumi's group (Takahashi *et al.*, 1982; Ohizumi *et al.*, 1983). Today the compound is being used extensively as a chemical tool in biomedical research.

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